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DOI:

[10.1002/chem.201801556](https://doi.org/10.1002/chem.201801556)

Document Version

Early version, also known as pre-print

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Citation for published version (APA):

Reading, E. (2018). Structural mass spectrometry of membrane proteins within their native lipid environments. CHEMISTRY. DOI: 10.1002/chem.201801556

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Going native: Structural mass spectrometry of membrane proteins within native environments

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Abstract: Mass spectrometry has emerged as an important structural biology tool for understanding membrane protein structure, function, and dynamics. Generally, structural mass spectrometry of membrane proteins has been performed on purified or reconstituted systems which lack the native lipid membrane and cellular environments. However, there has been progress in the use and adaptations of these methods for probing membrane proteins within increasingly more native contexts. In this Concept article the use and utility of structural mass spectrometry techniques for studying membrane proteins within native environments are highlighted.

Introduction

Membrane proteins are ubiquitous biological macromolecules which reside within dynamic and highly complex cellular membranes. The importance of membrane proteins, both in their diverse range of cellular functions and as key drug targets, makes understanding their fundamental processes a vital task. Membrane protein structural biology techniques characterise the structural arrangements and dynamics of membrane proteins to understand their function. Mass spectrometry (MS) has become an important method for membrane protein structural biologists due to its ability to determine a broad range of protein structural information, such as stoichiometry, subunit connectivity, structural dynamics, solvent accessibility, complex formation and ligand binding (**Figure 1**). The extensive capability of MS for the interrogation of membrane protein structure is afforded by the progress in a variety of structural MS-based approaches (whose historical and current developments have been excellently reviewed by others^[1]). These structural MS techniques include:

Native mass spectrometry (nMS)

nMS generates and transfers protein ions into the gas-phase using 'soft' ionisation processes, such as electrospray ionisation (ESI), nano-ESI, desorption ESI (DESI), and laser induced liquid bead ion desorption (LILBID), which retain their non-covalent interactions^[2]. An intact protein ion can then be detected and its mass determined, yielding information on membrane protein stoichiometry, complex formation, and ligand and lipid binding^[1b].

Chemical cross-linking mass spectrometry (XL-MS)

In XL-MS proteins are first labelled with a suitable bifunctional cross-linking agent, which covalently links one amino acid to another to form either intra- or inter-peptide crosslinks. The protein is then proteolysed and the crosslinked peptides identified and quantified by MS – the covalent nature of XL-MS affording considerable sample clean-up before MS analysis. XL-MS provides inter- and intra-protein proximities at a residue level of resolution. A wealth of information can be gained using XL-MS - the type of information dependent on the cross-linker agent used (e.g. protein-protein, protein-DNA, protein-RNA and/or protein-lipid crosslinkers) -informing on protein connectivity, structural dynamics, complex formation, solvent accessibility and the impact of ligand binding^[3].

Footprinting mass spectrometry

Footprinting MS involves the covalent amino acid modification of membrane proteins in solution using a suitable covalent labelling probe and/or process. The modified protein is then proteolysed and the site of these modifications, and their extent, detected using MS. These techniques are typically used to directly distinguish protein solvent accessibility but can also inform on conformational dynamics and ligand binding. Many covalent labelling strategies exist for membrane proteins, with fast photochemical oxidation of proteins (FPOP) being popular due to its ability to label several amino acids and its irreversible labelling enabling extensive post-labelling extraction and sample clean-up before MS analysis^[4]. FPOP is used to generate hydroxyl radicals ($\cdot\text{OH}$) which can react with the solvent accessible areas of a protein – these are generated either by synchrotron radiolysis of water or by controlled irradiation (at 248 nm) of a protein sample in the presence of small amounts of H_2O_2 and hydroxyl scavengers (e.g. glutamine and histidine). Other radical and non-radical chemical labelling strategies have also been used to report on membrane protein structure through specific or promiscuous amino acid labelling, for example, using carbene footprinting^[5], diethylpyrocarbonate (DEPC) labelling^[6], and cysteine specific N-ethylmaleimide (NEM) and lysine specific succinic anhydride labelling^[7].

Hydrogen/deuterium exchange mass spectrometry (HDX-MS)

HDX-MS is a type of footprinting technique which measures the extent and rate of exchange of protein backbone amide hydrogens for deuterium. In HDX-MS a membrane protein is exposed to deuterated buffer (or vice-versa) and quenched, left intact (global HDX) or proteolyzed (local HDX), and the mass-shifts and changes in the isotope distribution measured using MS. To minimize hydrogen/deuterium back-exchange during MS analysis, and therefore reduce structural information loss, the protein sample is typically quenched to acidic pH (typically pH 2.3 - 2.5) and sample clean-up and analysis performed rapidly at 0 °C. The rate or extent of hydrogen/deuterium exchange for backbone amides depends on structural features such as hydrogen bonding, solvent accessibility and protein conformational dynamics (either through structural fluctuations and/or unfolding events)^[8]. HDX-MS can provide both global and local (at both peptide and single amino acid resolution) information on protein structure, enabling membrane protein conformational dynamics, complex formation, solvent accessibility, and the impact of ligand binding and/or mutation on structure to be deciphered^[4].

Ion mobility spectrometry (IMS)

IMS can be coupled with MS to afford an additional time dimension for detected ions. Upon the influence of an electric field ions traverse through a drift-cell filled with an inert buffer gas, such as He or N₂, and are separated based on their differential mobility. IMS provides additional ion separation and improves MS signal-to-noise and peak capacity. It can also be used in combination with nMS to generate gas-phase shape information on intact proteins by determining their gas-phase collision cross-sections (CCSs)^[9].

Attaining membrane protein structural insight within more native environments would enhance our understanding of how a native membrane protein behaves and how they shape the function of cells, in both diseased and healthy states. Currently we acquire most of our understanding on membrane protein structure and dynamics *in vitro*, within highly purified membrane mimetic systems, such as detergent micelles, amphipols, bicelles and/or reconstituted lipid nanodiscs^[10]. However, within a cell the local environment of these membrane proteins is very different. Influences from cellular compartmentalization, crowding, local pH differences, and changes in membrane lipid composition and fluidity can have a significant influence on their function; likely because of changes in their structure and/or dynamics. Therefore, obtaining structural information within native environments must be a goal for membrane protein structural biologists.

Structural MS has the potential to obtain this information for membrane proteins. In this article, inspiring achievements within the field will be highlighted and their current limitations and future possibilities discussed, starting with the use of structural MS within rudimentary native environments and continuing to their use within the truest, the cell.

Basic but not simplistic: Native nanodiscs

Here, we consider the most basic native system to be a membrane protein retained within a lipid bilayer consisting of its native cellular lipid composition, where it has never been delipidated from its surrounding cellular lipid milieu. This can be achieved using the rapidly developing native nanodisc technologies^[11], which have become increasingly used in biochemical and structural biology investigations of membrane proteins^[12]. Native nanodiscs utilize an expanding repertoire of amphipathic polymers, styrene-maleic acid (SMA) being the most popular, to solubilize a membrane protein within its surrounding lipid bilayer directly from a cell membrane forming a 'native nanodisc' (Figure 2a). Importantly, the membrane protein

is contained within its native lipid bilayer, has never been in contact with potentially destabilizing detergents and/or has not been reconstituted into a lipid bilayer of non-native composition.

Recently, HDX-MS has been employed to study membrane proteins within native nanodiscs^[13]. Reading and colleagues successfully analysed the conformational dynamics of the *Escherichia coli* rhomboid protease, GlpG, within native nanodiscs (Figure 2). Sufficient sequence coverage of up to 80 % was achieved through optimization of quenching and digestion conditions, as well as the overall HDX-MS workflow. Moreover, the utility of native nanodiscs enabled the HDX structural interrogation of GlpG within different native lipid environments (generated using cell lines with differing lipid compositions or by altering cell growth temperatures during protein expression). From the HDX data general inferences could be made on the structural and dynamic behaviour of GlpG contained within a lipid bilayer of native constitution. Peptides within the transmembrane regions (TM) 2-6 of GlpG were protected from HDX, however, unpredictably, a part of the TM1 region linked to the cytosolic extension domain (CytD) of GlpG was found to be relatively unprotected to HDX and showed dynamic behaviour. The CytD and linker (Ln) regions of GlpG displayed much higher relative fractional deuterium uptake. Moreover, through comparison of HDX of GlpG within different native lipid environments (Δ HDX) the impact of alterations of native lipid composition on membrane protein conformational dynamics could be assessed. Changes in lipid headgroup composition did not seem to affect HDX of GlpG significantly, whereas changes in lipid chain length and degree of unsaturation did. Using HDX-MS, lipid sensitive regions were identified within the CytD, Ln and TM1 regions of GlpG, which were previously suggested to be important for protease substrate gating and/or function. It was proposed that these lipid sensitive regions could, therefore, play a functional role within GlpG through interactions with its membrane. More generally, this study validates the ability of HDX-MS to inform on membrane protein structure within well-defined native lipid environments.

Although native nanodiscs are certainly a valuable system for studying membrane proteins within native lipid environments they lack other cellular components, such as protein crowding, membrane curvature, transmembrane ion gradients, the native membrane phase and membrane protein topology. Nevertheless, successful adaptations of structural MS tools for studying membrane protein native nanodisc systems will undoubtedly lead to advances in our understanding of native membrane protein structure and function.

Increasing complexity: Membrane extracts and vesicles

Increasingly more native systems include isolated membrane preparations such as, membrane extracts, inside-out membrane vesicles and microvesicles. Isolated cellular membranes and vesicles contain membrane proteins within their natural lipid environment and can include the presence of other cellular proteins such as ribosomes, cytoplasmic proteins, and other peripheral and integral membrane proteins. Within vesicles a membrane protein will also experience membrane curvature and bilayer lateral pressure, as well as possess topology. These systems certainly provide environments more akin to the cellular one than native nanodiscs do - although their environments are much less defined - but also lack other characteristics of a cellular biological membrane, such as cellular crowding and their local cellular pH.

Membrane proteins have been studied within isolated biological membranes using structural MS, albeit not extensively. A pioneering study by Konermann and colleagues were the first to demonstrate that FPOP-MS could inform on membrane protein structure within a natural lipid environment (Figure 3)^[14]. They used bacteriorhodopsin (bR) contained within aqueous purple membrane suspensions as their model system (which contains ~75% (w/w) of bR, with the rest of the membrane consisting of neutral and acidic diether lipids). It was found that oxidative labelling was selective for methionine (Met) residues

within a membrane environment. This selectivity was suggested to be a result of lipids acting as efficient radical scavengers during FPOP, thus, preventing other (less amenable) amino acids being oxidatively modified. They determined that Met oxidation occurred at solvent accessible sites in bR (Met 32, 68 and 163) but not in membrane protected regions (Met 20, 56, 60, 118, 145 and 209). Interestingly, when comparing extents of Met oxidation of bR within its natural purple membrane environment to when in a denaturing sodium dodecyl sulfate (SDS) micellar environment they discovered increased solvent accessibility for Met residues in helix A (Met 20) and in helix D (Met 118), suggesting partial unfolding of these helices upon SDS denaturation^[4b]. In summary, Konermann and colleagues found that FPOP-MS can report directly on native membrane protein solvent accessibility and topology within its native membrane environment and, furthermore, demonstrated that it could be used to detect the occurrence of structural changes.

Another inventive FPOP-MS study by Yao and colleagues described the use of reversed footprinting FPOP-MS (which monitors the unoxidised peptides remaining after FPOP treatment instead of the presence of oxidised peptides) for the structural interrogation of membrane proteins within a native membrane^[15]. Using reversed FPOP-MS they examined the cystic fibrosis transmembrane conductance regulator (CFTR) protein within saponin semi-permeabilised baby hamster kidney cell membranes. The advantages of using this strategy include the simplification of data acquisition and analysis, as well as its capability to detect low-abundance peptides within complex environments. Using this approach, they could determine 'structural marker' peptides which potentially report on CFTR structure and decipher the presence of two channel populations (open and closed) of CFTR within its native plasma membrane.

In an impressive study by Konermann and colleagues, HDX-MS was used to probe the conformational and structural transitions of the F_0F_1 -ATP synthase during its catalytically driven rotation, within its natural membrane environment^[16]. By optimizing their HDX-MS workflow they could successfully

interrogate overproduced F_0F_1 -ATP synthase within isolated inside-out membrane vesicles (INVs) from *E. coli* using HDX-MS, enabling the influence of various catalytically active and inhibited conditions on its conformational dynamics to be explored. INVs have an internal and external environment contained within a biological membrane, this provides membrane protein topology and enables a proton motive force (PMF) to be established. Although HDX-MS sequence coverage was low for the membrane embedded subunits, sufficient protein coverage was achieved for the extramembraneous subunits (between 58-83% coverage); most of this region possessed similar HDX under different catalytic conditions, except for the C-terminal helix of the γ -subunit within the $\gamma\epsilon$ -rotor shaft which demonstrated substantial proton motive force (PMF) dependent HDX changes. Using their HDX data, and supporting biochemical, molecular dynamics and structural information from others, they could propose a model in which the γ -subunit undergoes load-dependent rotor destabilization in the ATP synthase molecular motor - akin to how bearings inflict greater forces on a crankshaft when operated under load within an automotive engine.

Overall, these studies demonstrate the power of structural MS to inform on native membrane protein structure within isolated cellular membranes, as well as determine functional and structural mechanisms of membrane proteins difficult to obtain with other techniques. However, these systems still do not capture the true native environment of a membrane protein, which is unquestionably their local environment within living cells. Although challenging, there has been intermittent success in the use of structural MS for the study of membrane protein structure within live cells.

The truest: the cell

XL-MS has been by far the most successful structural MS strategy to study membrane proteins within live cells and has even been extended to in-tissue studies^[17]. It has been widely used over the past few

decades with advances in cross-link chemistry, peptide enrichment and data analysis strategies significantly improving the amount of information which can be gained from an experiment^[3c]. Notably, these advances have led to proteome wide topological analysis and interaction networks of membrane proteins to be achieved^[18].

An impressive example of *in vivo* membrane protein XL-MS is the study of a several megadalton protein megacomplex utilized in cyanobacterium photosynthesis by Blankenship and Gross and colleagues^[19] (Figure 4). The megacomplex consists of the phycobilisome antenna complex (PBS) and the membrane protein photosystems I (PSI) and II (PSII); the light-harvesting antenna complex captures photons and transfers its energy to chlorophylls in photosystems I and II, where photochemical reactions take place. To understand the spatial orientations required for efficient energy transfer within these megacomplexes they used membrane-permeable chemical cross-linkers to capture weak interactions between the protein components within living cells. Using their XL-MS protocol they could determine exact interactions between the proteins within this megacomplex, enabling them to understand the structural arrangement of the megacomplex and infer on its functional mechanism. Additionally, using mono-link crosslinkers, which provide dead-end cross links, they could also decipher solvent accessibility information on proteins within the megacomplex.

Although *in vivo* XL-MS undoubtedly provides information on native membrane protein structure and its interactions, it is currently limited in its ability to confidently decipher membrane protein conformational dynamics and solvent accessibility *in vivo*. In-cell footprinting MS and/or HDX-MS could potentially yield this information. HDX-MS has not yet been used for *in vivo* analysis of membrane proteins, however, it has been successfully utilized for *in organello* studies. Forest and Pelosi and colleagues used HDX-MS to explore the conformational dynamics of an adenine nucleotide carrier membrane protein within bovine mitochondria (bAnc1p)^[20]. Using the specific transport inhibitors carboxyatractyloside (CATR) and

bongkreikic acid (BA) they could fix bAnc1p into two different conformations and use HDX-MS to identify its conformational dynamics within mitochondria. Although protein coverage was limited (29 peptides detected, 58% coverage), they could distinguish structurally informative differences between the two inhibitor-bound systems and, interestingly, when comparing to their previous work on detergent solubilised bAnc1p found stark differences in comparative HDX for the upper half of the carriers' cavity. These differences were hypothesised to be due to the influence of the native membrane environment and led to the revaluation of their previously proposed functional mechanism for bAnc1p. This study highlights the power of HDX-MS, and its potential importance, for deciphering native functional mechanisms of membrane proteins upon ligand binding.

Excitingly, footprinting MS has been successful in providing information on membrane proteins *in vivo*; the efficacy of in-cell footprinting MS using the FPOP technology being recently pioneered by Jones and colleagues^[21]. It was demonstrated that existing FPOP-MS strategies (Figure 3a) could be adapted for in-cell footprinting analysis of both soluble and membrane proteins. In-cell footprinting was achieved by incubating live African green monkey kidney (Vero) cells with low levels of H₂O₂ (which can readily diffuse across cell membranes) followed by FPOP labelling and quenching using cell permeable radical scavengers, such as, dimethylthiourea and N-tert-butyl- α -phenylnitrone. 105 oxidatively modified endogenous proteins were found upon MS analysis with a large number (~30 %) of these proteins being modified membrane proteins detected from various organelles. Importantly, they validated that in-cell FPOP can monitor solvent accessibility as shown by a high degree of correlation found between the extent of the oxidative residue modification and the solvent accessible surface areas of actin. While there was evidence of some cell death and/or reduction in the functional capacity of the live cells upon exposure to H₂O₂ - as well as evidence of labelling-induced structural artefacts during FPOP^[22] - in-cell FPOP still promises to be an exciting structural MS tool for studying membrane protein solvent accessibility and conformation within its native cellular environment.

Concluding remarks

In this Concept article the potential capability of structural MS for deciphering membrane protein behaviour within native environments has been highlighted. Even with these examples, with the possible exception of XL-MS, the widespread use of structural MS for the study of membrane proteins within more complex and biologically relevant systems are few and far between. This is likely due to the inherent hydrophobicity of membrane proteins and lipids which lead to difficulties in sample clean-up and handling. Additionally, many of the membrane protein structural MS procedures currently require laborious manual analytical procedures and data analysis, and/or the requirement for specialized equipment and expertise. This discourages their adoption by other laboratories in academia and industry. Many of these limitations have been addressed in structural MS, especially in the development of software for improved data analysis and increasingly higher throughput methods. Yet, there is still a lack of general analytical methods for studying membrane proteins within native environments using many of the structural MS techniques discussed in this article.

XL-MS is leading the line currently due to its larger scientific community and, thus, more extensive use in structural biology, both as a standalone technique and as an exceptional hybrid tool with techniques such as X-ray crystallography, cryo-EM and NMR^[23]. XL-MS technique(s) will likely continue to be a major contributor to understanding native, cellular membrane protein structure. Other structural MS techniques need to catch-up because they can offer different and/or complementary information to XL-MS, enabling a more developed picture of native membrane protein function and structure to be achieved. This is particularly important as hybrid MS has established ways to combine data from multiple structural MS and molecular modelling techniques to build highly informative models of protein structure and function^[24]. Impressive achievements in the use of footprinting techniques, such as HDX-MS and FPOP-MS, have been presented in this article which forecasts a promising future for their continued

development and success in this field, where the demonstration of nMS and nMS-IMS of membrane proteins within native nanodiscs is likely forthcoming.

More generally, membrane protein structural MS methods offer the potential for examining ligand, drug and lipid interactions with membrane proteins, in well-defined and complex native lipid environments. An exciting prospect which will impact on the continued and developing use of structural MS for membrane protein structural biology and drug discovery^[25].

Acknowledgements

Dr Eamonn Reading is funded by a BBSRC Future Leader Fellowship (BB/N011201/1). I acknowledge Dr. Arygyris Politis, Dr. Chloé Martens and Zainab Ahdash (King's College London) for critical reading of the manuscript.

Conflict of Interest

The author declares no conflict of interest.

Keywords: Membrane proteins • Mass spectrometry • Structure-activity relationships • Protein structure
• Protein dynamics

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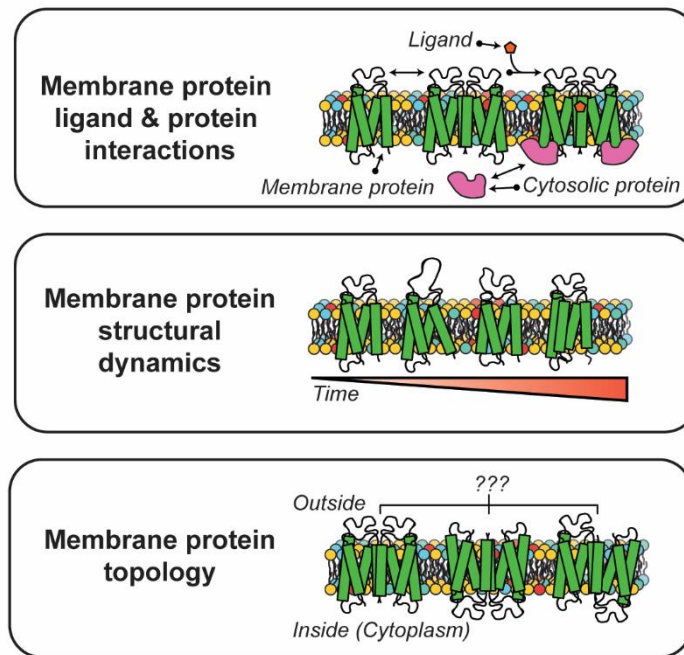


Figure 1. Membrane protein structural information obtainable using structural MS techniques.

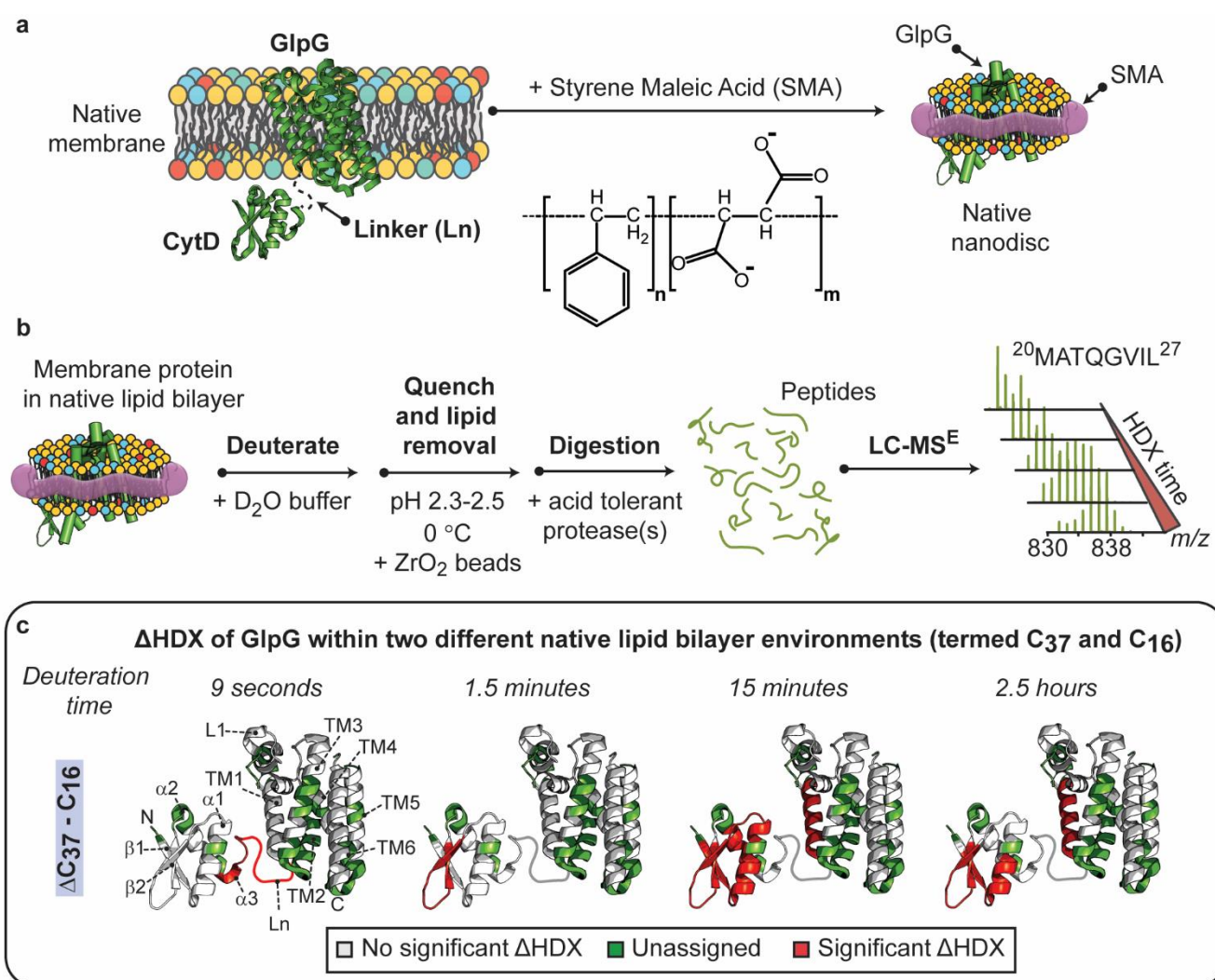


Figure 2. Studying membrane protein conformational dynamics within native nanodiscs with HDX-MS. **(a)** Schematic of styrene maleic acid (SMA) membrane protein extraction from a native membrane into native nanodiscs (SMALPs), which consists of its native lipid milieu. The rhomboid protease GlpG (PDB: 2XTV and 2LEP) is used as an example. **(b)** A generalized workflow for HDX-MS of membrane protein within native nanodiscs. **(c)** HDX-MS analysis of GlpG within two different native lipid environments. C₃₇ and C₁₆ systems contain similar abundances (mol %) of phospholipid type (phosphatidylethanolamines (PE), phosphatidylglycerols (PG) and cardiolipins (CDL)): C₃₇ has 62 ± 4 % PE, 36 ± 4 % PG, and 2.0 ± 0.7 % CDL and C₁₆ has 64 ± 3 % PE, 36 ± 3 % PG, and 0.3 ± 0.1 % CDL. There were significant differences in fatty acid chain unsaturation and small differences in chain length between the two systems – C₁₆ possessed higher abundances of unsaturated chains and a small increase in longer chain length quantity compared to C₃₇. Monitoring the temporal differences in HDX (ΔHDX) of the GlpG membrane protein within the two different native lipid systems revealed regions that were conformationally sensitive

to alterations to their native lipid environment. Parts of this figure was previously published by Wiley VCH in Reading *et al*^[13].

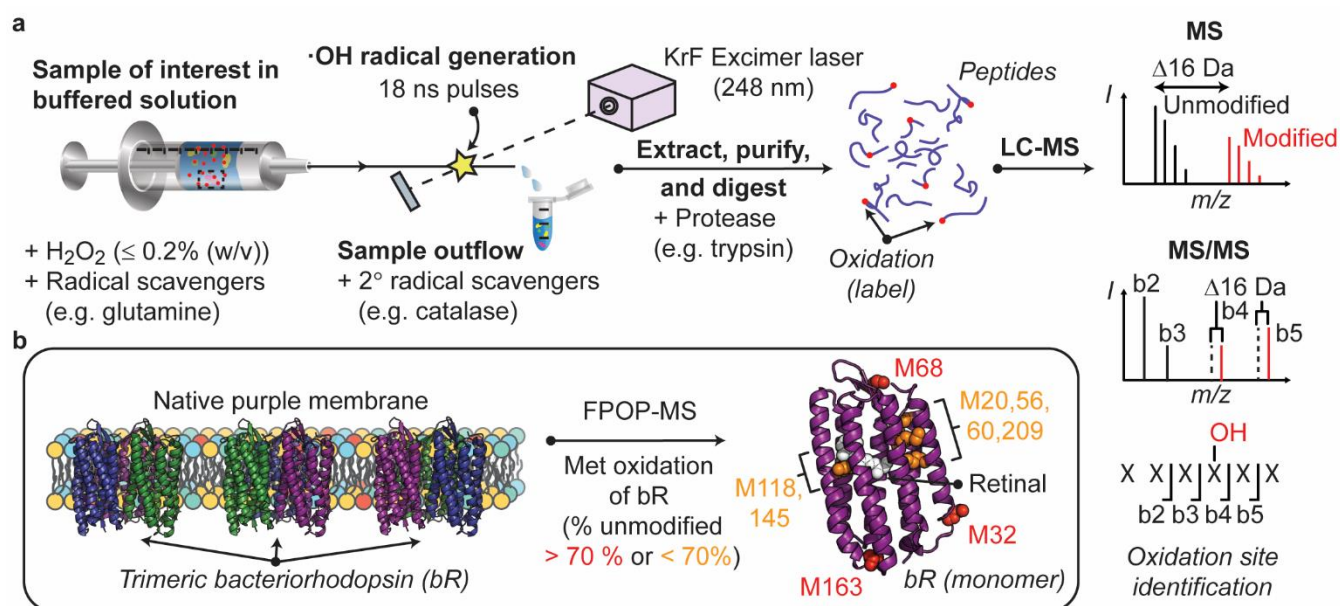


Figure 3. Footprinting MS of a membrane protein within a native environment using the fast-photochemical oxidation of proteins (FPOP) method. **(a)** A generalized workflow for membrane protein FPOP-MS within a native environment, including within live cells. **(b)** FPOP-MS of bacteriorhodopsin (bR) within its native purple membrane revealed that the extent of methionine (Met/M) residue oxidation correlates with the solvent accessibility and topology of native bR structure^[4b, 14].

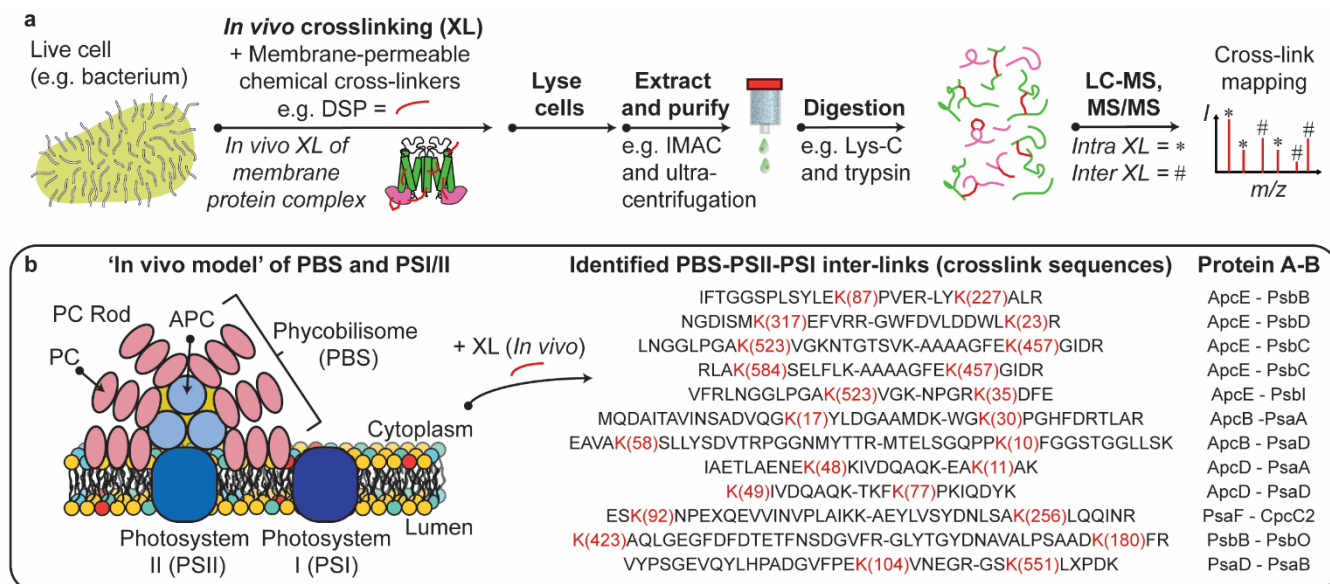
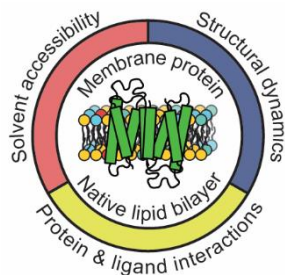


Figure 4. *In vivo* crosslinking MS (XL-MS) of membrane protein complexes. **(a)** A generalized workflow of *in vivo* XL-MS. **(b)** *In vivo* XL-MS using the membrane permeable lysine (K) crosslinker dithiobis[succinimidy]propionate] (DSP) revealed inter crosslinks between the individual components within the several megadalton megacomplex from the cyanobacterium *Synechocystis* PCC 6803 (inter lysine crosslinks are in red). These inter crosslinks enabled the native, cellular molecular arrangement of the megacomplex to be modelled and understood, providing a basis for understanding how the phycobilisomes (PBS) transfer excitation energy to reaction centres and how the energy balance of two photosystems (photosystem I (PSI) and II (PSII)) is achieved. The '*in vivo* model' and inter crosslink information was replicated from Liu *et al.*^[19].

Table of Contents



Natural behaviour: Membrane proteins have evolved to perform their functions within the setting of a cell membrane. Structural mass spectrometry methods can explore the structure and dynamics of membrane proteins within their native cellular membrane environments.